

FORM PTO-1390 (REV. 11-2000)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER 58049-00002	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				U.S. APPLICATION NO. (If known, see 37 CFR 1.5 10/088548	
INTERNATIONAL APPLICATION NO. PCT/KR99/00554		INTERNATIONAL FILING DATE September 15, 1999		PRIORITY DATE CLAIMED September 15, 1999	
TITLE OF INVENTION A NOVEL ANGIOGENESIS INHIBITOR					
APPLICANT(S) FOR DO/EO/US CHANG, Jihoon; KIM, Jang Seong; PARK, Eun Jeong; YUM, Jung-sun; CHUNG, Soo-il					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
<p>1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.</p> <p>4. <input checked="" type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31).</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))</p> <p>a. <input checked="" type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau).</p> <p>b. <input type="checkbox"/> has been communicated by the International Bureau.</p> <p>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</p> <p>6. <input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).</p> <p>a. <input type="checkbox"/> is attached hereto.</p> <p>b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4).</p> <p>7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))</p> <p>a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau).</p> <p>b. <input type="checkbox"/> have been communicated by the International Bureau.</p> <p>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</p> <p>d. <input type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p> <p>Items 11 to 20 below concern document(s) or information included:</p> <p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input checked="" type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input checked="" type="checkbox"/> A FIRST preliminary amendment.</p> <p>14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment</p> <p>15. <input type="checkbox"/> A substitute specification.</p> <p>16. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>17. <input checked="" type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.</p> <p>18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4)</p> <p>19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).</p> <p>20. <input checked="" type="checkbox"/> Other items or information: Microorganism Deposit Receipt; International Search Report</p>					

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Express Mail Label No. EL 824098099 US

1071210/088548

1010 Rec'd PCT/PTO 15 MAR 2002 PATENT 5804500002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Jihoon Chang et al.)	Group Art Unit: Not Yet Determined
)	
Serial No.: Not Assigned Yet)	Examiner: Not Yet Determined
)	
Based On PCT/KR99/00554 filed on Sep. 15, 1999)	
)	
Filed: March 15, 2002)	
)	
For: A NOVEL ANGIOGENESIS INHIBITOR)	
)	

PRELIMINARY AMENDMENT, SUBMISSION OF SEQUENCE LISTING, AND

SUBMISSION OF MICROORGANISM DEPOSIT RECEIPT

BOX SEQ
Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to examination of the above-identified application on the merits, please amend the application as follows:

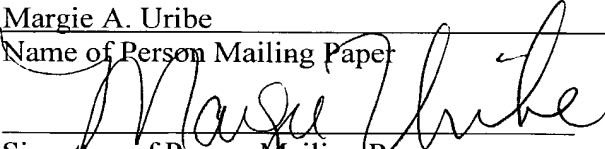
CERTIFICATE OF MAILING
(37 C.F.R. § 1.10)

I hereby certify that this paper (along with any referred to as being attached or enclosed) is being deposited with the United States Postal Service on the date shown below with sufficient postage as "Express Mail Post Office to Addressee" in an envelope addressed to Box Patent Application, Commissioner for Patents, Washington, D.C. 20231.

EL 824098099 US
Express Mail Label No.

March 15, 2002
Date of Deposit
LosAngeles/75109.1

Margie A. Uribe
Name of Person Mailing Paper


Signature of Person Mailing Paper

IN THE SPECIFICATION:

At page 1, line 3, add the following:

-- CROSS REFERENCE TO OTHER APPLICATIONS

The present application is filed under 35 U.S.C. 371, and is the U.S. national phase application of PCT/KR99/00554, filed on September 15, 1999. --

IN THE CLAIMS:

Please amend claims as follows:

1. (Amended) LK6 protein comprising amino acid sequences of human apolipoprotein(a) kringle domains IV36.
2. (Amended) LK7 protein comprising amino acid sequences of human apolipoprotein(a) kringle domains IV37.
3. (Amended) LK8 protein comprising amino acid sequences of human apolipoprotein(a) kringle domains V38.
4. (Amended) LK68 protein comprising amino acid sequences of human apolipoprotein(a) kringle domains IV36, IV37 and V38 in a serial manner
5. (Amended) A cDNA sequence which codes for the LK6 protein of claim 1.

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6. (Amended) A cDNA sequence which codes for the LK7 protein of claim 2.
7. (Amended) A cDNA sequence which codes for the LK8 protein of claim 3.
8. (Amended) A cDNA sequence which codes for the LK68 protein of claim 4.

REMARKS

Claims 1-19 are pending in the application. The amendments to the claims serve to further clarify the present invention. No new matter has been inserted into the application. Accordingly, entry of the amendments to the application and examination on the merits are respectfully requested.

Submission Of Sequence Listing

Enclosed herewith in full compliance with 37 C.F.R. §§ 1.821-1.825 is a Sequence Listing to be inserted into the specification. The Sequence Listing in no way introduces new matter into the specification.

Also submitted herewith in full compliance with 37 C.F.R. §§ 1.821-1.825 is a computer readable disk copy of the Sequence Listing. Applicant's undersigned representative hereby states that the information recorded in computer readable form is identical to the written sequence listing.

Submission of Microorganism Deposit Receipt

Applicants submit herewith the deposit receipts for *Escherichia coli* BL21/LK6-8 (KCTC 0633BP) and *Escherichia coli* BL21/LK8 (KCTC 0634BP) which were deposited with the Korean Collection for Type Cultures on June 9, 1999, and *Escherichia coli* BL21(DE3)/LK6 (KCTC

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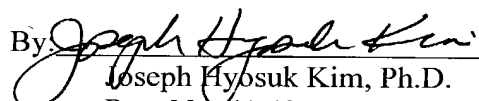
0655BP) and *Escherichia coli* BL21(DE3)/LK7 (KCTC 0656BP) which were deposited with the Korean Collection for Type Cultures on Sept. 3, 1999. Applicants hereby declare that subject to paragraph (b) of 37 CFR 1.808, all restrictions imposed by the depositor on the availability to the public of the deposited material will be irrevocably removed upon the granting of the patent.

The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 07-1853 during the pendency of prosecution of this application. A duplicate of this paper is enclosed for the Deposit Account, should it be needed.

Respectfully submitted,

SQUIRE, SANDERS & DEMPSEY L.L.P.

Dated: March 15, 2002

By 
Joseph Hyosuk Kim, Ph.D.
Reg. No. 41,425

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VERSION MARKED TO SHOW CHANGES MADE

In the Specification

At page 1, line 3, add the following:

-- CROSS REFERENCE TO OTHER APPLICATIONS

The present application is filed under 35 U.S.C. 371, and is the U.S. national phase application of PCT/KR99/00554, filed on September 15, 1999. --

In the Claims

Please amend claims 1-8 as follows:

1. (Amended) LK6 protein [(SEQ ID NO: 4) consisting of] comprising amino acid sequences of human apolipoprotein(a) kringle domains IV36.
2. (Amended) LK7 protein [(SEQ ID NO: 6) consisting of] comprising amino acid sequences of human apolipoprotein(a) kringle domains IV37.
3. (Amended) LK8 protein [(SEQ ID NO: 8) consisting of] comprising amino acid sequences of human apolipoprotein(a) kringle domains V38.
4. (Amended) LK68 protein [(SEQ ID NO: 2) consisting of] comprising amino acid sequences of human apolipoprotein(a) kringle domains IV36, IV37 and V38 in a serial manner

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5. (Amended) A cDNA sequence [(SEQ ID NO: 3)] which codes for the LK6 protein of claim 1.

6. (Amended) A cDNA sequence [(SEQ ID NO: 5)] which codes for the LK7 protein of claim 2.

7. (Amended) A cDNA sequence [(SEQ ID NO: 7)] which codes for the LK8 protein of claim 3.

8. (Amended) A cDNA sequence [(SEQ ID NO: 1)] which codes for the LK68 protein of claim 4.

SEQUENCE LISTING

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10/088548

JC10 Rec'd PCT/PTO 15 MAR 2002
A NOVEL ANGIOGENESIS INHIBITORBACKGROUND OF THE INVENTION

5

Field of the Invention

The present invention relates to a novel angiogenesis inhibitor, LK68 whose amino acid sequence is identical with the human apolipoprotein(a) kringle domains IV36, IV37 and V38, more specifically, to an amino acid sequence of the LK68, a cDNA sequence encoding the LK68, a recombinant expression vector comprising the cDNA, a recombinant microorganism transformed with the recombinant expression vector and a novel use of the LK68 as an anticancer agent and a method for treating the angiogenesis-mediated disease.

Description of the Prior Art

20

Angiogenesis is a biological process of generating new blood vessels into a tissue or organ. Under normal physiological conditions, humans or animals undergo angiogenesis only in very specific restricted situations. For example, angiogenesis is normally observed in wound healing, fetal and embryonal development and formation of the corpus luteum, endometrium and placenta. It has been reported that new vessel growth is tightly controlled by many angiogenic regulators (see: Folkman, J., Nature Med., 1: 27-31, 1995a), and the switch of the angiogenesis phenotype depends on the net balance between up-regulation of angiogenic stimulators and down-regulation of angiogenic suppressors.

An imbalance of the angiogenic process has been shown to contribute to pathological disorders such as diabetic retinopathy, rheumatoid arthritis and psoriasis (see: Folkman, J., Nature Med., 1: 27-31, 1995a).

Especially, both primary and metastatic tumors need to recruit angiogenic vessels for their growth (see: Folkman, J., New Engl. J. Med., 285:1182-1186, 1971; Folkman, J., J. Biol. Chem., 267:10931-10934, 1992). If
5 this angiogenic activity could be repressed or eliminated, then the tumor, although present, would not grow. There are many reports suggesting that inhibiting tumor angiogenesis should provide a practical approach to long term control of the disease. Blocking positive
10 regulators of angiogenesis or utilizing negative regulators to suppress angiogenesis results in a delay or regression of experimental tumors. If the angiogenic activity could be repressed or eliminated, then the tumor, although present, would not grow. Moreover, in
15 the disease state, prevention of angiogenesis could avert the damage caused by the invasion of the new microvascular system effectively. Therefore, therapies directed at control of the angiogenic process could lead to the abrogation or mitigation of these diseases.

20 Therefore, what is needed is a novel angiogenesis inhibitor which can inhibit the unwanted growth of blood vessels, especially into tumors. An anticancer agent comprising the angiogenesis inhibitor should be able to overcome the activity of endogenous growth factors in
25 premetastatic tumors and prevent the formation of the capillaries in the tumors thereby inhibiting the growth of the tumors. The anticancer agent should also be able to modulate the formation of capillaries in other angiogenic processes, such as wound healing and
30 reproduction. Finally, the anticancer agent and method for inhibiting angiogenesis should preferably be non-toxic and produce few side-effects.

Until now, at least 10 endogenous angiogenic inhibitors have been identified in the art (see:
35 O'Reilly, M. S. et al., Cell, 88: 277-285, 1997). One such molecule is angiostatin, which consists of the plasminogen kringle I through IV(see: O'Reilly, M. S.

- et al., Cell, 79:315-328, 1994). When applied systemically, angiostatin powerfully inhibits both primary tumor growth and metastasis without toxicity, and angiogenesis induced by bFGF as well (see: O'Reilly, M. S. et al., Nature Med., 2:689-692, 1996). These anti-tumor effects were accompanied by a marked reduction of microvessel density within the tumor mass, indicating that suppression of angiogenesis was associated with the inhibition of tumor growth.
- 10 Kringles are protein structural domains composed of approximately 80 amino acids and three intramolecular disulfide bonds. Kringle structures are found in many proteins such as prothrombin (see: Walz, D. A. et al., Proc. Natl. Acad. Sci., U.S.A., 74:1969-1973, 1977),
- 15 plasminogen(see: Ponting, C. P., Blood Coagul. & Fibrinolysis, 3:605-614, 1992), urokinase(see: Pennica, D. et al., Nature, 301:579-582 1983), hepatocyte growth factor(see: Lukker, N. A. et al., Protein Eng., 7:895-903, 1994), and apolipoprotein("apo")(a) (see: McLean, J. W. et al., Nature, 330:132-137, 1987). These domains appear to be independent folding units, but their functional role is not yet known. The previous reports represent that the kringle structure can act as inhibitors of endothelial cell migration and
- 25 proliferation during angiogenesis. Specifically, prothrombin's kringle 2 and plasminogen's kringle 1-4, and 5 have been shown to be anti-angiogenic(see: Ji, W. R. et al., FASEB J., 15:1731-1738, 1998a; Ji, W. R. et al., Biochem. Biophys. Res. Commun., 247:414-419, 1998b; Cao, Y. et al., J. Biol. Chem., 271:29461-29467, 1996; Cao, Y. et al., J. Biol. Chem., 272:22924-22928, 1997; Barendsz-Janson, A. F., J. Vasc. Res., 35:109-114, 1998; Lee, T. H. et al., J. Biol. Chem., 273:28805-28812, 1998).
- 30
- 35 Apolipoprotein(a), one of the proteins having kringle structures, is a candidate for a novel angiogenesis inhibitor. Apo(a) is covalently attached to

apoB-100, the main protein component of low density lipoprotein(LDL) to form lipoprotein(a)(see: Fless, G. M., J. Biol. Chem., 261: 8712-8718, 1986). Elevated plasma concentration of Lp(a) represents a major
5 independent risk factor for arteriosclerosis(see: Armstrong, V. W. et al., Artherosclerosis, 62:249-257, 1986; Assmann, G., Am. J. Cardiol., 77:1179-1184, 1996; Bostom, A. G. et al., JAMA, 276:544-548, 1996). Although several pathogenic activities have been reported, the
10 physiological role of apo(a) has not yet been established(see: Lawn, R. M. et al., J. Biol. Chem., 271:31367-31371, 1996; Scanu, A. M. and Fless, G. M., J. Clin. Invest., 85:1709-1715, 1990; Utermann, G., Science, 246:0904-910, 1989).

15 Apo(a) contains two types of kringle domains and an inactive protease-like domains: the first 37 kringle domains are ~75% identical to plasminogen kringle IV, and the last kringle domain is 90% identical to plasminogen kringle V. Interestingly, the kringle IV-
20 like domain is present in 15-40 copies in different human alleles of the apo(a) gene. In this regard, it is feasible to develop an inhibitor of tumor angiogenesis and growth employing the Apo(a) kringle structures.

25 SUMMARY OF THE INVENTION

In accordance with the present invention, the inventors have cloned and expressed the human apo(a) kringles containing IV36, IV37 and V38 as a recombinant
30 protein LK68, and discovered that: the LK68 protein and its single kringles, LK6, LK7 and LK8, have an ability to overcome the angiogenic activity of endogenous growth factors such as bFGF *in vitro*; and they may be used as active ingredients of anticancer agents.

35

The first object of the invention is, therefore, to provide a novel LK68 protein consisting of human apo(a)

kringle domains IV36, IV37 and V38, and cDNA encoding the LK68 protein.

The second object of the invention is to provide a novel recombinant vector containing the cDNA encoding human apo(a) kringle domains IV36, IV37 and V38.

The third object of the invention is to provide an anticancer agent which comprises the LK68 protein or its single kringles, LK6, LK7 and LK8, as an active ingredient.

The fourth object of the invention is to provide a method for treating angiogenesis-mediated disease by employing the LK68 protein.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and the other objects and features of the present invention will become apparent from the following description given in conjunction with the accompanying drawings, in which:

Figure 1 is a photograph of a SDS-polyacrylamide gel electrophoresis for analysis of recombinant LK68 protein expressed in *E. coli*.

Figure 2 is a photograph showing the inhibition of angiogenesis by LK68 on the chick chorioallantoic membrane (CAM).

Figure 3(A) is a graph showing inhibition of vessel growth in the CAM as a function of LK68.

Figure 3(B) is a graph showing inhibition of vessel growth in the CAM as a function of single kringles, LK6, LK7, LK8, and a control.

- Figure 4(A) is a graph showing inhibition of BCE cell proliferation by recombinant LK68 and angiostatin.
- 5 Figure 4(B) is a graph showing inhibition of BCE cell proliferation by recombinant LK6, LK7 and LK8.
- 10 Figure 4(C) is a graph showing inhibition of HUVEC cell proliferation by recombinant LK68 and LK8.
- 15 Figure 5(A) is a graph showing BrdU labeling index of LLC cells in the presence of recombinant LK68 and LK8.
- 20 Figure 5(B) is a graph showing BrdU labelling index of Y1 cells in the presence of recombinant LK68 and LK8.
- 25 Figure 5(C) is a graph showing BrdU labelling index of TIB74 cells in the presence of recombinant LK68 and LK8.
- Figure 5(D) is a graph showing BrdU labelling index of CHO cells in the presence of recombinant LK68 and LK8.
- 30 Figure 5(E) is a graph showing BrdU labelling index of MSF cells in the presence of recombinant LK68 and LK8.
- 35 Figure 5(F) is a graph showing BrdU labelling index of NIH3T3 cells in the presence of recombinant LK68 and LK8.

Figure 6(A) is a graph showing inhibition of HUVEC cell migration by recombinant LK68, LK8 and PK5.

5 Figure 6(B) is a graph showing inhibition of HUVEC cell migration by recombinant LK68, LK6, LK7 and LK8.

10 Figure 7 is a graph showing inhibition of BCE cell migration by angiostatin, recombinant LK68, LK6, LK7, and LK8 and combination of single kringles.

15 Figure 8 shows the effect of administration of LK68 to mice having implanted Lewis lung carcinoma cells on total volume as a function of time.

20 Figures 9(A) to 9(C) are photographs showing histological analyses of Lewis lung carcinoma cells by hematoxylin and eosin (H/E) staining.

25 Figure 10 shows the effect of administration of LK68 to nude mice having implanted human lung carcinoma A549 cells on total volume as a function of time.

30

DETAILED DESCRIPTION OF THE INVENTION

35 The present invention provides a novel protein LK68, which can be cloned and expressed as recombinant protein from the human apolipoprotein("apo")(a) kringles. The LK68 protein consists of amino acid sequences of human

apolipoprotein(a) kringle domains IV36(amino acid 8 to 80), IV37(amino acid 122 to 194) and V38(amino acid 226 to 300) in a serial manner(see: SEQ ID NO: 2). The first two kringle domains of LK68 (i.e., IV36 and IV37) are
5 homologous to human plasminogen kringle IV, and the third kringle domain V38 is homologous to human plasminogen kringle V. The present invention also provides a cDNA encoding the LK68 protein (see: SEQ ID NO: 1) and recombinant vectors which comprises the said
10 cDNA and expression vectors such as pET vector series.

In describing the kringle domains of the invention, human apolipoprotein(a) kringles IV36, IV37 and V38 are abbreviated as KIV36, KIV37 and KV38, respectively; LK68 is employed to mean the recombinant protein which
15 comprises the said three kringle domains; and, LK6, LK7 and LK8 are employed to mean the recombinant proteins of KIV36, KIV37 and KV38, respectively.

Because apolipoprotein(a) contains plasminogen-type IV and V kringle domains, it was assumed that
20 apolipoprotein(a) could possibly have an anti-angiogenic activity. There is an experimental evidence suggesting apolipoprotein(a) may contain biological activity as an inhibitor of tumor angiogenesis and growth(see: Trieu, V. N. and Uckun, F. M., Biochem. Biophys. Res. Commun.,
25 257:714, 1999). It has been reported that LL/2(Lewis Lung Carcinoma) tumor growth is delayed in apo(a) transgenic mice and the microvessel density of LL/2 tumors from apo(a) transgenic mice is lower than that from wild-type mice as control.

30 Under the circumstance, the present inventors assumed that LK68 protein, its single kringles or their functional equivalents may have an anti-angiogenic activity. To verify said anti-angiogenic activity, it was investigated whether recombinant LK68 and its single
35 kringles (i.e., LK6, LK7 and LK8) are potent anti-angiogenic factors *in vitro* and *in vivo* as well. As a result, LK68, LK6, LK7 and LK8 exhibit inhibitory

activities on the cultured endothelial cell proliferation as well as on the endothelial cell migration. LK68 and its single kringles also inhibit the normal development of capillaries in the chick embryo chorioallantoic membrane (CAM). It was also shown that systemic administration of LK68 inhibited the primary tumor growth, which is correlated with a suppression of tumor-induced angiogenesis. Since each of the single kringle proteins, LK6, LK7 and LK8 showed anti-angiogenic activity, it is expected that they also inhibit the primary tumor growth or metastasis.

Accordingly, LK68 protein, its single kringles or their functional equivalents may be applied for the development of a potent anti-cancer agent, which is highly effective for angiogenesis-mediated diseases covering rheumatoid arthritis, psoriasis, or ocular angiogenic diseases, etc.

Also, LK68 protein, its single kringles or their functional equivalents may be used in combination with other compositions and procedures for the treatment of diseases. For example, tumor may be treated conventionally with surgery, radiation or chemotherapy combined with LK68, its single kringles, or their functional equivalent, and then LK68, its single kringles, or their functional equivalent may be subsequently administered to the patient to extend the dormancy of micrometastases and to stabilize and inhibit the growth of any residual primary tumor.

The present invention is further illustrated in the following examples, which should not be taken to limit the scope of the invention.

Example 1: Cloning and Expression of Recombinant LK68

In order to verify the anti-angiogenic activity of human apo(a) kringle, the inventors cloned and expressed

the last three kringles containing IV36, IV37 and V38 as a recombinant protein LK68. A DNA fragment of apo(a) spanning nucleotides 12,052 to 12,975 (see: McLean J. W. et al., Nature, 330:132, 1987) was PCR-amplified from human liver cDNA and the resulting 924-bp *NdeI*-*BamHI* fragment was ligated into *E.coli* expression vector pET11a(Novagen, USA). The oligonucleotide primers A(SEQ ID NO: 9) and F(SEQ ID NO: 14) (see: Table 1) were used for PCR amplification under the standard PCR protocol. This clone was named "pET11a/LK68", which encodes 308 amino acids including human apo(a) kringle domains, IV36, IV37 and V38 (see: SEQ NO ID: 2). The first two kringle domains of this clone, IV36 and IV37, are homologous to human plasminogen kringle IV, and the third kringle domain V38 is homologous to human plasminogen kringle V.

The nucleotide sequences of this clone were confirmed in both directions. When the nucleotide sequence of this clone was compared to the same region of the human apo(a) (see: McLean J. W. et al., Nature, 330:132, 1987), the nucleotide sequences are identical with the exception of a single base change at nucleotide 554. Our clone contains a cytosine at this position as compared to a thymidine in the sequence reported by McLean et al. (see: McLean J. W. et al., Nature, 330:132, 1987), causing an amino acid change to Thr from Met. This substitution has also been reported by other groups (see: Van der-Hoek, Y. Y. et al., Hum. Mol. Genet., 2:361-366, 1993; LoGrasso, P. V. et al., J. Biol. Chem., 269:21820-21827, 1994) and appears to be the predominant allele for apo(a).

E. coli BL21(DE3) was transformed with an expression plasmid pET11a/LK68 and recombinant LK68 protein was expressed under the following conditions. One liter of Luria-Bertani broth containing ampicillin was inoculated with 10ml of an overnight culture of *E. coli* BL21(DE3) harboring the pET11a/LK68 plasmid and incubated with shaking at 37°C. When the OD₆₀₀ of the

culture reached 0.4-0.6, isopropylthio- β -D-galactoside(IPTG) was added at a final concentration of 1mM. Cells were grown an additional 4h after induction. Cells were harvested by centrifugation at 8000xg for
5 30min at 4°C. These cell pellets were sonicated and the over-expressed proteins were analyzed by SDS-PAGE(see: Figure 1). In Figure 1, Mr represents a molecular weight marker(Boehringer Mannheim, Germany); lane 1, the expression of recombinant LK68 protein without IPTG
10 induction; and, lane 2, the expression of recombinant LK68 protein with IPTG induction, respectively. Recombinant LK68 protein having a molecular weight of 37kDa was well expressed in *E. coli*, accumulating to about 20-30% of the total protein, as evidenced by image
15 analysis of the scanned gel. The transformant thus prepared was designated as '*Escherichia coli* BL21/LK6-8', and deposited with the Korean Collection for Type Cultures, #52 Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea, an international depository authority
20 as accession No. KCTC0633BP on Jun. 9, 1999.

Each of single kringle domains, IV36, IV37 and V38, was cloned separately into an expression vector pET15b as described above. The oligonucleotide primers used for
25 cloning are listed in Table 1: that is, A(SEQ ID NO: 9) and D(SEQ ID NO: 12) for KIV36 cloning; B(SEQ ID NO: 10) and E(SEQ ID NO: 13) for KIV37 cloning; and, C(SEQ ID NO: 11) and F(SEQ ID NO: 14) for KV38 cloning, respectively. These three couples of oiligonucleotide
30 primers were used for PCR amplification under the standard PCR protocol and the resulting clones were named "pET15b/LK6", "pET15b/LK7" and "pET15b/LK8", each of which includes the single human apo(a) kringle domains of IV36, IV37 and V38, respectively. *E. coli*
35 BL21(DE3) competent cells were transformed with each of the expression plasmid, pET15b/LK6, pET15b/LK7 and pET15b/LK8. The transformant with plasmid pET15b/LK6

* Restriction sites, *Nde*I and *Bam*HI are added for the cloning

To assess if LK68 was expressed in the soluble fraction or the insoluble cellular fraction of *E.coli* cells, the inventors analyzed the LK68 expression in these fractions. This analysis showed that LK68 was located in the insoluble cellular fraction. Thus, it was necessary to denature, refold and reoxidize the disulfied bonds of LK68. By using the deoxycholate and other detergents, the insoluble LK68 protein was purified as inclusion bodies to the extent of >95% purity. Then, the inclusion bodies were solublized with 7M urea and folded into native conformation using a rapid dilution and an equilibrium dialysis scheme. In the folding buffer, purified inclusion bodies were easily refolded without detectable protein aggregation. After the dialysis, the protein was purified by lysine-Sepharose 4B affinity chromatography. The protein bound to lysine-Sepharose was specifically eluted by ϵ -ACA(ϵ -amino-n-caproic acid). This suggested that the lysine-

binding site located in the KIV37 kringle of the refolded protein was fully functional. Affinity elution of LK68 with 0.1M ϵ -ACA yielded about 3mg of protein/g of cells(wet weight). Chromatography with polymyxin-B
5 beads(Sigma Chemical Co., USA) was subsequently performed to eliminate any endotoxin, and residual endotoxin activity was determined with the *Limulus* amebocyte lysate assay kit(Biowhittaker Inc., USA). The purified protein was analyzed by SDS-PAGE and was stored
10 at -20°C until needed. The calculated pI value of LK68 protein is 6.13. The N-terminal amino acid sequence of the purified LK68 was confirmed by amino acid sequencing.

Example 3: Chick Chorioallantoic Membrane Assay

15 In order to determine whether LK68 is anti-angiogenic *in vivo*, the inventors tested its ability to inhibit the development of capillaries in the chorioallantoic membrane("CAM")(see: Lee, T. H. et al.,
20 J. Biol. Chem., 273:28805-28812, 1998). Fertilized three-day-old eggs were incubated at 37°C, and a window was made after the extraction of ovalbumin. After two days of incubation, a Thermanox coverslip(Nunc Inc., USA) containing recombinant LK68 protein was applied to
25 the CAM of individual embryos. After 48h, 20% fat emulsion was injected into the chorioallantois of the embryos, and the vessel formation around the Thermanox was examined(see: Figure 2). In Figure 2, the left photograph shows the normal development of capillaries
30 in the CAM; and, the right shows the inhibition of angiogenesis by LK68 on CAM, respectively.

When LK68 at the dose range of 3 - 5 μ g was applied on the CAM, more than 60 % among the 100 eggs tested showed avascular zone around the sample applied,
35 indicating that the growth of capillaries was inhibited. With the recombinant proteins of each kringle domain, e.g. LK6, LK7 or LK8, 60 - 70% of the eggs tested showed

inhibitory effects at the dose range of 1µg/CAM(see: Figures 3(A) and 3(B)). This in vivo study showed that apo(a) kringle domains have anti-angiogenic activity and LK68 as well as single kringle proteins is a potent
5 inhibitor of angiogenesis. There was no evidence of toxicity in any of the chick embryos tested.

Example 4: Inhibition of Endothelial Cell Proliferation

10 Recombinant LK68, LK6, LK7 and LK8 proteins were assayed for their inhibitory activity on proliferation of bovine capillary endothelial (BCE) cells stimulated by bFGF under the following conditions. BCE cells were grown in DMEM containing 10% bovine calf serum (BCS) and
15 3 ng/ml bFGF(Upstate Biotechnology, USA). Approximately 3,000 cells were added to each well of 96-well tissue culture plate and incubated at 37 °C in 5% CO₂ atmosphere. After incubation for 18 h, the medium was replaced with DMEM containing 0.5% BCS, and the test samples were
20 added to each well. After 30 min incubation, bFGF was added to a final concentration of 1ng/ml. The cell count was determined by [³H]thymidine incorporation method. The experiments were performed in triplicate.

As can be seen in Figure 4, it was determined that
25 LK68, LK6, LK7 and LK8 specifically inhibited BCE cell proliferation in a dose-dependent manner. When the angiostatin was applied as a positive control, all the Apo(a) kringle proteins tested appeared to be more effective under the conditions used in this experiment.
30 The concentration of half-maximal inhibition (ED₅₀) for LK68 is determined about 200 - 250nM, about 140 - 170nM for LK6, about 10 - 20nM for LK7, and about 10 - 20nM for LK8 (see: Figures 4(A) and 4(B)).

Recombinant LK68 and LK8 proteins were assayed for
35 their inhibitory activity on proliferation of human umbilical vein endothelial (HUVEC) cells stimulated by bFGF under the following conditions. HUVECs(American

Type Culture Collection, USA) were grown in F12K medium containing 10% heat-inactivated fetal bovine serum("FBS")(Hyclone, USA), 30 μ g/ml endothelial cell growth supplement(ECGS)(Sigma Chemical Co., USA), and
5 100 μ g/ml heparin(Sigma Chemical Co., USA). The cells were plated at a density of 2000/well in 96-well tissue culture plate. The cells were incubated at 37°C, 5% CO₂, for 18hr, washed once with serum-free medium, and F12 medium containing 0.5% FBS was added. The cells were
10 treated with various concentrations of samples and incubated for 30min. Then, ECGS, heparin and bFGF(Upstate Biotechnology, USA) were added into the cells with the final concentrations of 30 μ g/ml, 100 μ g/ml and 5ng/ml, respectively. After 48hr of incubation,
15 cell counts were determined with the Cell Proliferation ELISA using 5-bromo-2'- deoxyuridine (BrdU)(Boehringer Mannheim, USA). The experiments were performed in triplicate.

As can be seen in Figure 4(C), it was determined
20 that LK68 as well as LK8 specifically inhibited HUVEC cell proliferation in a dose-dependent manner.

In the presence of LK68 or single kringle proteins such as LK6, LK7 and LK8, the morphology of BCE or HUVEC cells appeared similar to those of untreated cells. In
25 addition, cell proliferation can be rescued with bFGF stimulation after removal of LK68. These results indicate that LK68 as well as single kringle proteins are not cytotoxic to capillary endothelial cells. Furthermore, the inhibitory activity would appear to be
30 specific for endothelial cells, e.g., BCE and HUVEC cells. Additionally, LK68 as well as LK8 failed to show inhibition of proliferation of non-endothelial cell types, such as CHO cells, mouse skin fibroblast NIH3T3 cells, mouse Lewis lung carcinoma cells, mouse adrenal
35 tumor Y1 cells and mouse embryonic liver/SV40 transformed cell line TIB74(see: Figures 5(A) to 5(F)). Figures 5(A) to 5(C) represent the sensitivity of

various tumor cells such as LLC, Y1, and TIB 74, and Figures 5(D) to 5(F) represent the sensitivity of various normal cell lines such as CHO, MSF, and NIH3T3, respectively.

5

Example 5: Inhibition of Endothelial Cell Migration

Cell migration assay was performed in Transwells with 8-mm pores (Costar, USA). Briefly, the wells were
10 coated with fibronectin(25 μ g/ml)(Sigma Chemical Co., USA) overnight and HUVECs were plated at a density of 2000/well in 100 μ l Dulbecco's modified Eagle's medium containing 0.4% fetal calf serum(FCS) in the upper chamber. 500 μ l of DMEM containing 0.4% FCS was added to
15 the lower chamber and incubated at 37°C for 1 hr. The test samples of 1 μ M concentration were added to the upper chamber and 25 ng/ml of bFGF was added to the lower chamber. After 5 hr incubation, cells that crossed the fibronectin-plated membrane were quantified after
20 wiping off the cells in the upper chamber with a cotton swab. The cells across the membrane were stained with Diff-Quik stain set according to the manufacturer's instruction (Dade Behring Inc., USA) and were counted at 100x magnification. The experiments were performed in
25 duplicate.

Basic FGF(25ng/ml) was used to stimulate the migration of HUVEC cells. With the dose of 1 μ M, LK68 as well as single kringle proteins such as LK6, LK7 and LK8 completely inhibited the bFGF-induced HUVEC cell
30 migration to the level of uninduced control(see: Figures 6(A) and 6(B)). In Figure 6, (-)CON represent uninduced control, and (+)CON represent bFGF-induced positive control.

Migration assay using BCE cells was performed as
35 described above. Two different concentrations of LK68 or single kringle proteins applied and all the Apo(a) kringle proteins tested showed inhibitory effects on BCE

cell migration. In addition, LK68 and its single kringle proteins were more effective on the inhibition of BCE cell migration than angiostatin(AS) (see: Figure 7).

5 Example 6: Suppression of Primary Tumor Growth

Example 6-1: Lewis Lung Carcinoma

Male 6 to 8-week-old C57BL6/J mice were implanted
10 with Lewis lung carcinomas. The subcutaneous dorsa of mice in the proximal midline were injected with 1×10^6 cells in 0.1ml of saline. When the tumors reached about 5mm in diameter, tumor-bearing mice received LK68(100 mg/kg body weight) as a suspension in PBS injected
15 subcutaneously at a site distant from the tumor. The control group of mice had only a sham procedure and was treated with PBS only. Tumor size was measured every day during the treatment; and, volumes were determined using the formula $\text{width}^2 \times \text{length} \times 0.52$ and the ratio of
20 treated to control tumor volume(T/C) was determined for the last time point. Treatments were continued for 8 days, at which point all mice were sacrificed and the tumors were removed(see: Figure 8). As can be seen in Figure 8, it was clearly determined that the growth of
25 LLC primary tumors was potently suppressed by systemic LK68 therapy; LK68 at a dose of 100mg/kg caused significant regression of tumor burden only with 7 day treatment.

Histological analyses were also carried out to
30 compare tumors from treated and control mice in terms of vessel density and hemorrhage formation, and morphological appearance(see: Figures 9(A) to 9(C)). In Figures 9(A) to 9(C), 9(A) shows PBS-treated control, 9(B) LLC tumors of 10mg/kg body weight LK68-treated, and
35 9(C) LLC tumor of 100mg/kg body weight LK68-treated, respectively. Obvious histological differences were observed in LK68-treated tumors by hemotoxylin and

eosin(H/E) staining: that is tumor cells were not intact and morphologically not viable; and, zonal necrosis was examined around the tumors. Also, vessel density within LK68-treated tumors was reduced. There was no evidence
5 of inflammation or bleeding in any of the mice treated with the recombinant LK68.

Example 6-2: Human Lung Carcinoma

10 Four-week-old outbred female *nu/nu* nude mice used in this experiment were housed in a sterile environment. Cages, bedding, food and water were all autoclaved. The mice were maintained on a 12-hr light/ 12-hr dark cycle. Human lung cancer cells (A549 purchased from Korean Cell
15 Line Bank) were maintained in RPMI 1640 medium, supplemented with 10% heat-inactivated FBS and antibiotics. Approximately 2×10^7 cells of A549 human lung carcinoma were subcutaneously injected into nude mice into the proximal midline of the dorsa. When tumors
20 were palpable at day 7 after tumor implantation, the mice were treated with LK68 at the dose of 100mg/kg body weight. The control group was treated with PBS only. The treatment was continued for 17 days. The tumor size was measured every other day.

25 The tumor growth was regressed by the LK68 treatment: that is, LK68-treated A549 tumors were approximately 57.5% smaller than tumors in control animals(see: Figure 10). There was no evidence of any toxicity in any of the treated mice. Continued therapy
30 maintained the tumors in a state of dormancy for as long as it was administered. These data strongly suggest that the anti-angiogenic effect of LK68 can be used to target a wide variety of primary malignancies.

35 As clearly illustrated and demonstrated as above, the present invention provides a novel angiogenesis inhibitor, LK68 whose amino acid sequence is identical

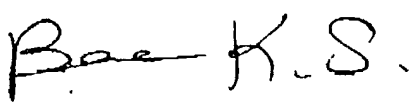
BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT
OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

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TO: Mogen Biotechnology Research Institute
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Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: <i>Escherichia coli</i> BL21(DE3)/LK6	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCTC 0355BP
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by <input checked="" type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on Sep 03 1999 .	
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The microorganism identified under I above was received by this International Depositary Authority on _____ and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on _____.	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
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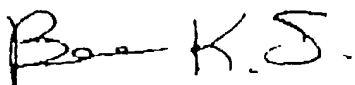
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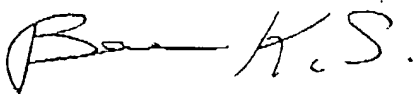
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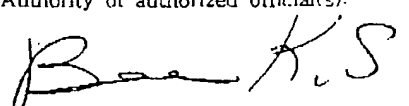
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11. A recombinant expression vector pET15b/LK8 comprising the cDNA of claim 7 which expresses the LK8 protein of claim 3.

5

12. A recombinant expression vector pET11a/LK68 comprising the cDNA of claim 8 which expresses the LK68 protein of claim 4.

10 13. *Escherichia coli* BL21(DE3)/LK6(KCTC0655BP) transformed with the recombinant expression vector pET15b/LK6 of claim 9.

15 14. *Escherichia coli* BL21(DE3)/LK7(KCTC0656BP) transformed with the recombinant expression vector pET15b/LK7 of claim 10.

20 15. *Escherichia coli* BL21/LK8(KCTC0634BP) transformed with the recombinant expression vector pET15b/LK8 of claim 11.

25 16. *Escherichia coli* BL21/LK6-8(KCTC0633BP) transformed with the recombinant expression vector pET11a/LK68 of claim 12.

25

17. An anticancer agent which comprises an active ingredient of LK68 protein, its single kringles, or their functional equivalents and pharmaceutically acceptable carrier.

30

18. A method for treating angiogenesis-mediated disease which comprises administering therapeutically effective amount of LK 68 protein, its single kringles, or their functional equivalents to a human or animal.

35

19. The method for treating angiogenesis-mediated disease of claim 18, wherein the angiogenesis-mediated

disease is cancer, rheumatoid arthritis, psoriasis, or ocular angiogenic disease.

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ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: A NOVEL ANGIOGENESIS INHIBITOR

(57) Abstract: The present invention provides a novel angiogenesis inhibitor, LK68 whose amino acid sequence is identical with the human apolipoprotein (a) kringle domains IV36, IV37 and V38, a cDNA sequence encoding the LK68, a recombinant expres-
sion vector comprising the cDNA, a recombinant microorganism transformed with the recombinant expression vector and a novel
use of the LK68 as an anticancer agent and a method for treating angiogenesis-mediated disease. LK68, LK6, LK7 and LK8 ex-
hibit inhibitory activities on the cultured endothelial cell proliferation as well as on the endothelial cell migration. LK68 and its
single kringles also inhibit the normal development of capillaries in the chick embryo chorioallantoic membrane (CAM). It was also
showed that systemic administration of LK68 causes the inhibition of primary tumor growth, which is correlated with a suppression
of tumor-induced angiogenesis. Accordingly, LK68 protein, its single kringles or their functional equivalents may be applied for the
development of a potent anti-cancer agent, which is highly effective for angiogenesis-mediated diseases covering cancer, rheumatoid
arthritis, psoriasis, ocular angiogenic disease, etc.

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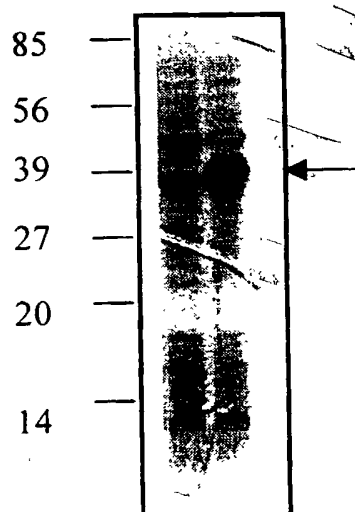


FIG. 1

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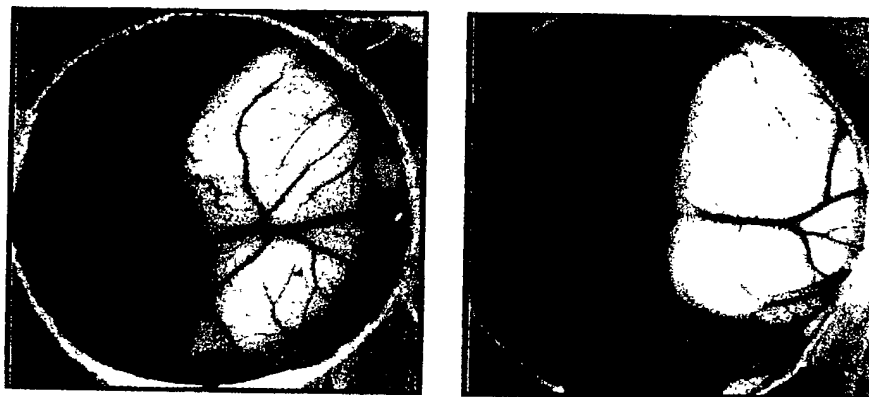


FIG. 2

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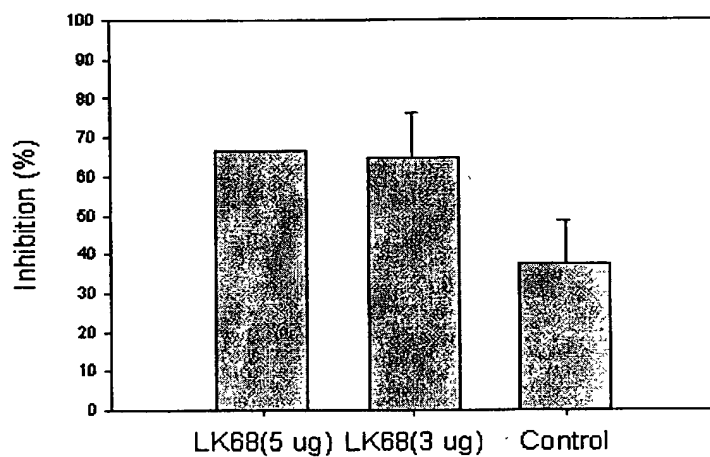


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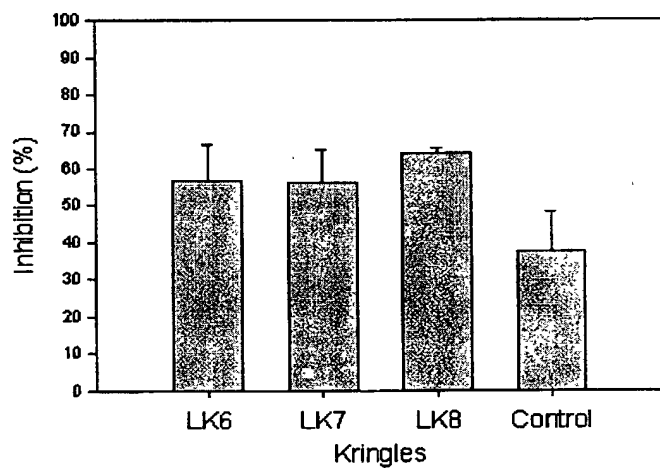


FIG. 3 (B)

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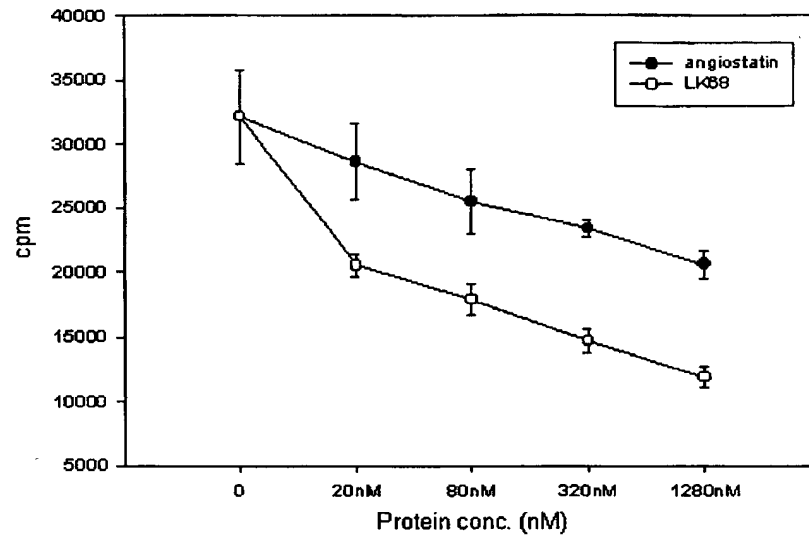


FIG. 4 (A)

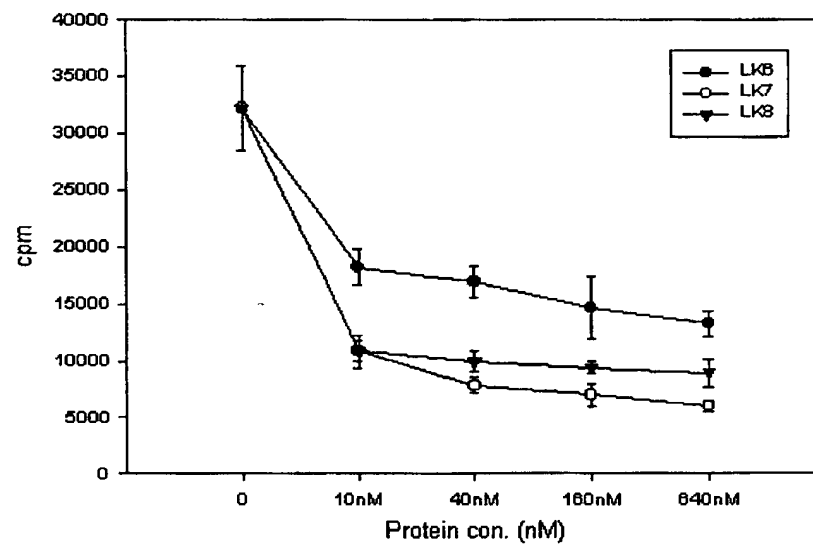


FIG. 4 (B)

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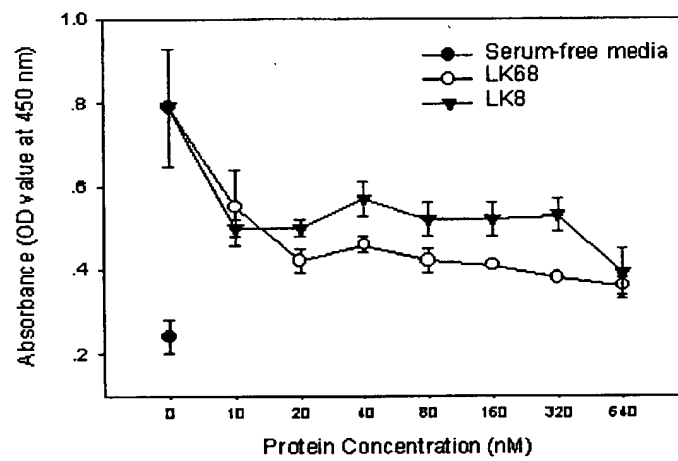


FIG. 4 (C)

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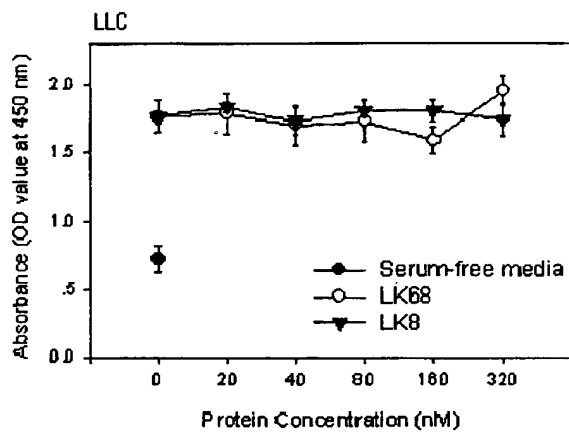


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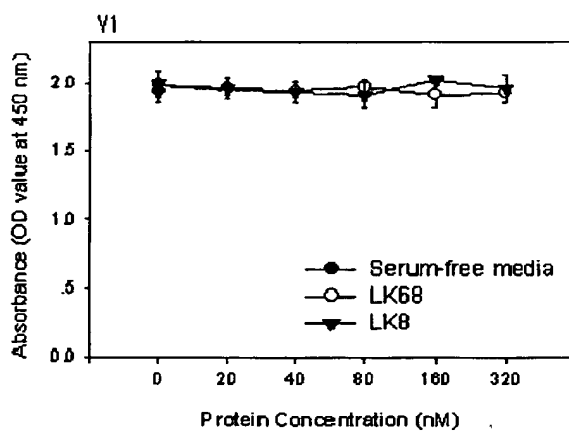


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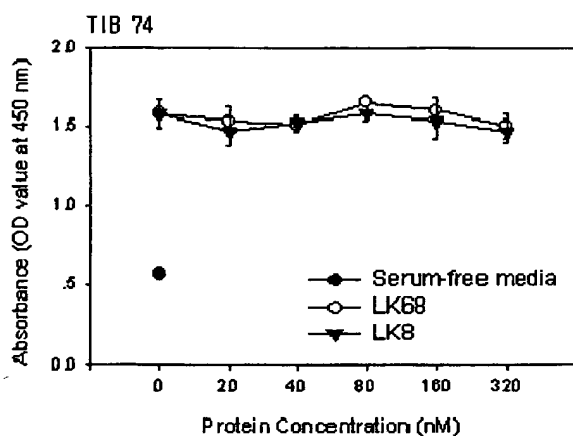


FIG. 5 (C)

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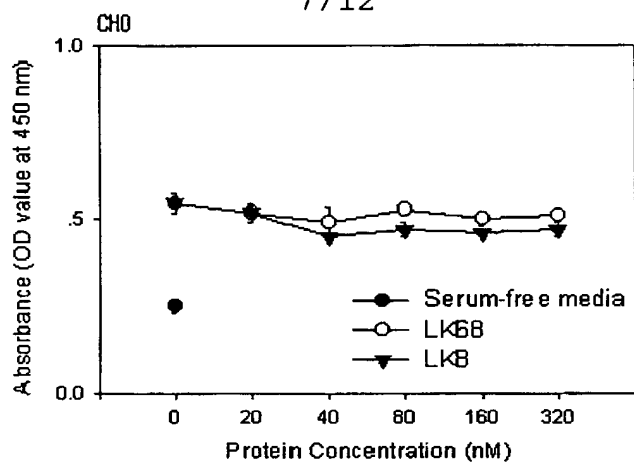


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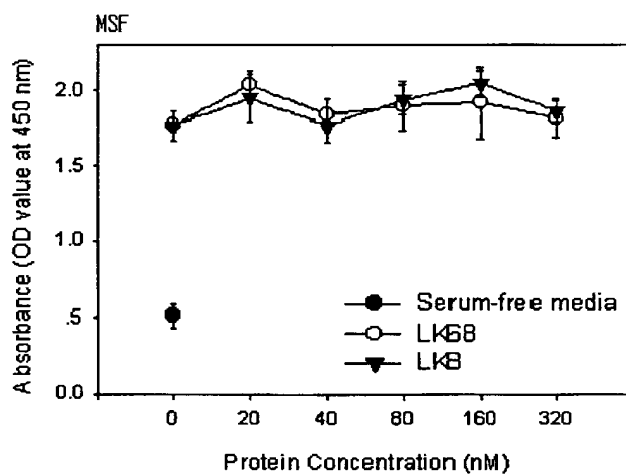


FIG. 5 (E)

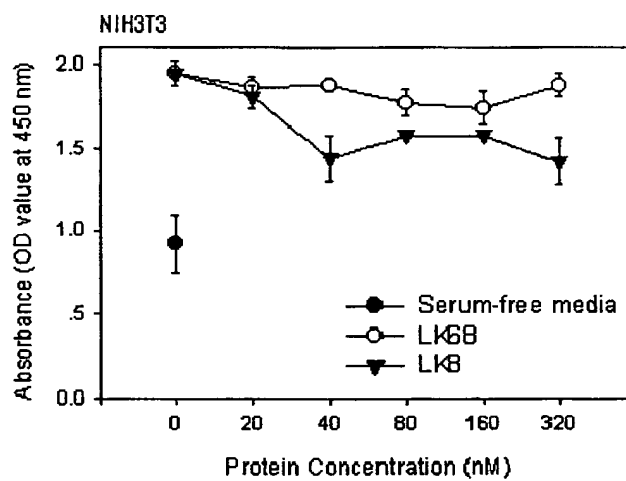


FIG. 5 (F)

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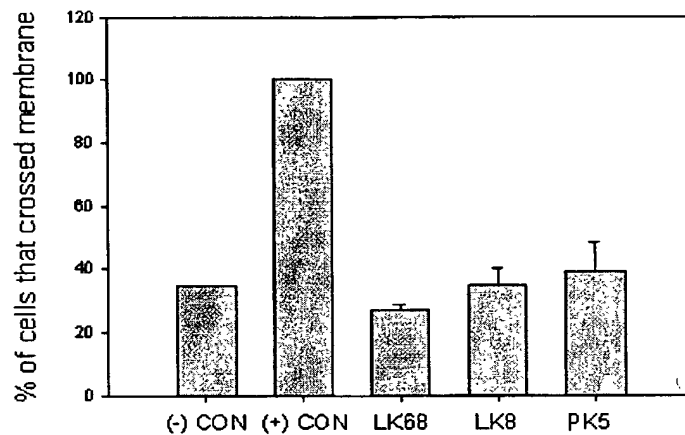


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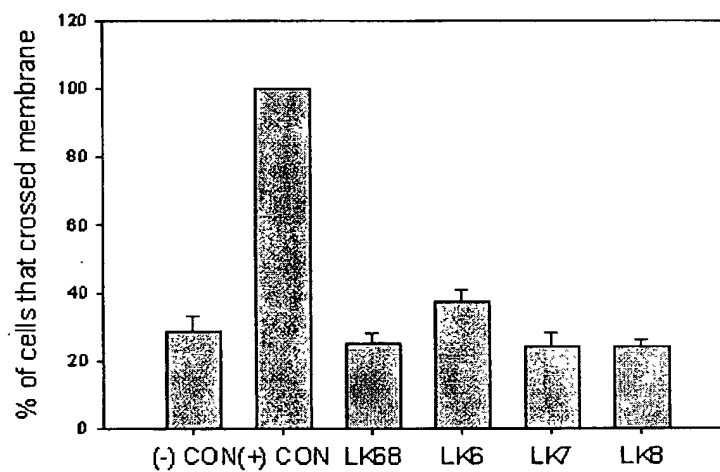


FIG. 6 (B)

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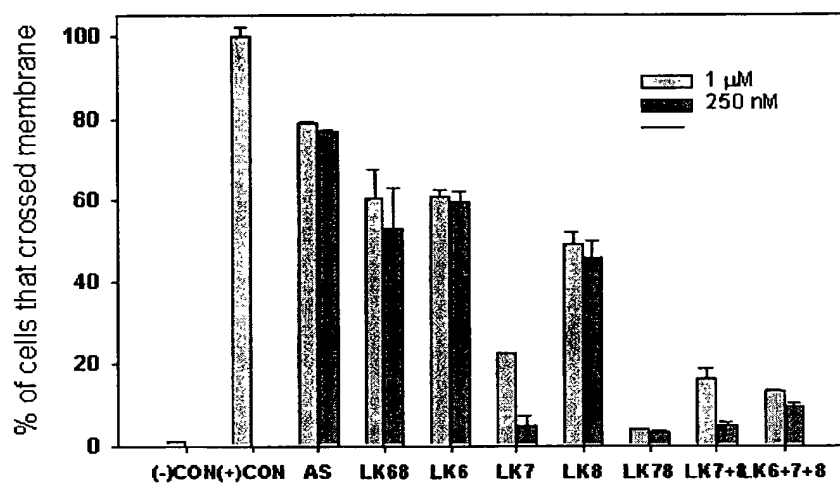


FIG. 7

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Suppression of LLC by LK68

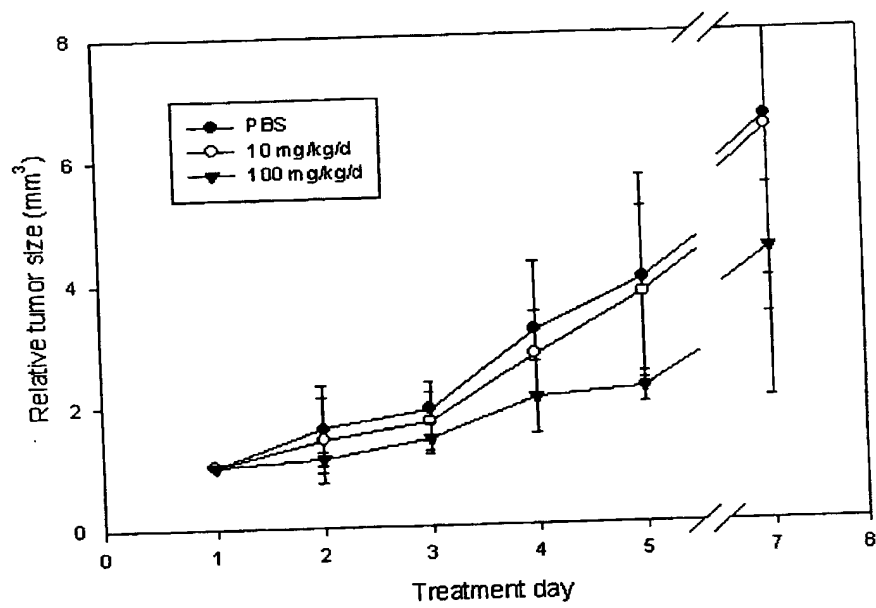


FIG. 8

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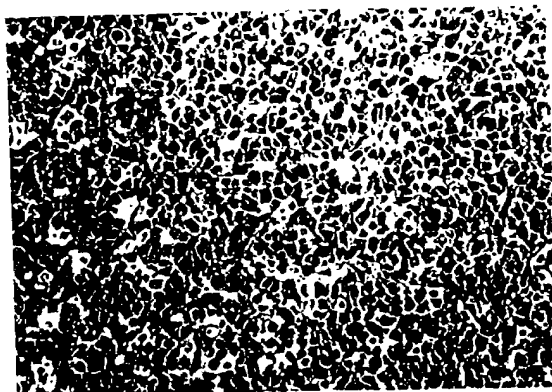


FIG. 9(A)

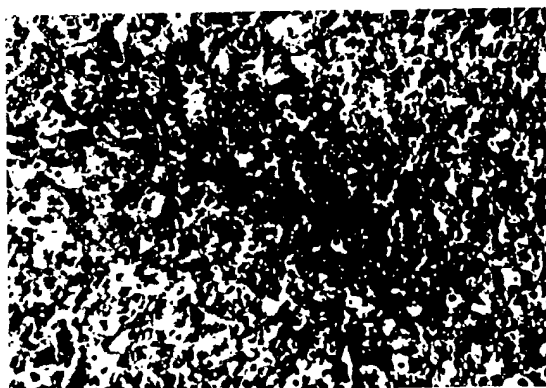


FIG. 9(B)



FIG. 9(C)

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Tumor suppression by LK 6-8 (hlung carcinoma)

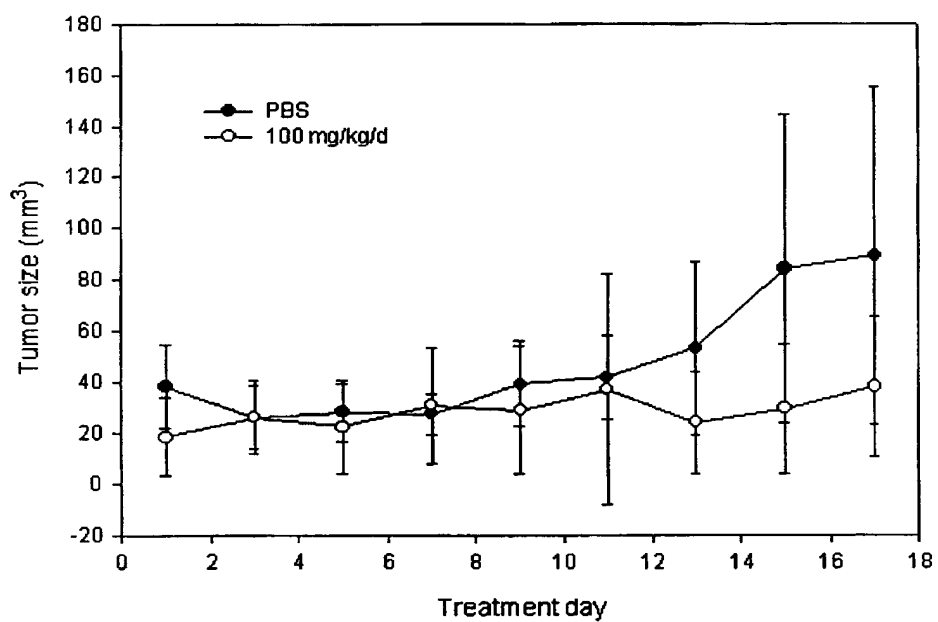


FIG. 10

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Patent
58049-00002**DECLARATION AND POWER OF ATTORNEY**

As a below named inventor, I hereby declare:

THAT my residence, post office address and citizenship are as stated below next to my name.

THAT I believe I am the original, first and sole (if only one name is listed below) or an original, first and joint inventor (if plural inventors are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: **A NOVEL ANGIOGENESIS INHIBITOR** the specification of which:

☒ [X] is attached hereto.

OR

☐ [] was filed on _____, as United States Application Number _____

THAT the subject matter of the

☐ [] attached amendment

OR

☐ [] amendment filed on (MM/DD/YY)

was part of my or our invention and was invented before the filing date of the original application, above identified for such invention.

THAT I do not know and do not believe that this invention was ever known or used in the United States of America before my or our invention or discovery thereof, or patented or described in any printed publication in any country before my or our invention or discovery thereof, for more than one year prior to this application.

THAT the invention was not in public use or on sale in the United States of America for more than one year prior to this application.

THAT this invention has not been patented or made the subject of an inventor's certificate issued before the date of the application in any country foreign to the United States of America on an application filed by me or my legal representatives or assigns more than twelve months before this application.

THAT I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

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THAT I acknowledge the duty to disclose information of which I am aware which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations §1.56.

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Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YY)	Priority Claimed?		Certified Copy Attached?	
			YES	NO	YES	NO
PCT/KR99/00554	PCT	September 15, 1999	X			X

THAT I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YY)

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U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YY)	Parent Patent Number (If applicable)

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And as a named inventor, I hereby appoint the following registered practitioners to prosecute this application and to transact all business in the United States Patent and Trademark Office connected therewith and with the resulting patent, individually and collectively:

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I hereby declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

10087543 031592

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Patent
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SEQUENCE LISTING

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